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Approaches to the genetics of cardiovascular disease through genetic field work

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Approaches to the genetics of cardiovascular disease through genetic field work. Successful molecular genetic studies of complex disease require exact, careful phenotypization, which is more difficult than that performed for monogenic diseases. We have developed a family-oriented field working approach, which relies on index patients, their primary care physicians, and a minimum number of field working staff. The patients are responsible for recruiting their family members. Packets containing an explanatory pamphlet, an informed consent statement, a questionnaire, and blood cuvettes are provided. Data are transferred from questionnaire and from the laboratory into a computer program that facilitates construction of the family tree. We have applied this genetic field working approach primarily to patients with lipid disorders. Coupling results from genetic field working with modern DNA diagnostic tests such as the oligonucleotide ligation assay, has enabled us to effectively identify patients with familial hypercholesterolemia in the German population. We are now extending genetic field working to hypertension. Hypertension is much more difficult to study, because the phenotype is more difficult to discern and document. Both complex diseases have the disadvantage that the parents of the index patients are likely to already be dead. Nevertheless, we concentrate on the recruitment of large pedigrees, sibling pairs with parents whenever possible, and trios consisting of index patient and both parents or index patient, parent and sibling. With these constellations we can conduct association studies, linkage analysis, and novel combinations of both approaches.

Patients at high risk for cardiovascular disease are generally identified by the presence of risk factors, such as lipid disturbances, hypertension, diabetes mellitus, or elevated fibrinogen values [1, 2]. A positive family history is a well recognized predictor, since lipid patterns, blood pressure values, type II diabetes mellitus, and coagulation disturbances are all strongly influenced by genetic variance [3, 4]. Genetically attuned physicians and geneticists engaged in scientific research have developed a variety of strategies aimed at defining heritability as well as at identifying new genes involved in complex polygenic diseases [5, 6]. Both linkage and novel association studies rely on recruitment of families whose phenotype must be carefully defined. Family studies permit both linkage analyses with both affected sib-pair and family data, as well as sophisticated association approaches such as haplotype sharing or haplotype relative risk [7]. Less appreciated is the important opportunity the index patient's family represents in providing comprehensive preventative medical care. Obtaining a family history is routine for primary care physicians, however, the family history is usually only a cursory exercise and receives at best a few lines in the patient's medical records. Family members of patients with complex, polygenic diseases warrant medical attention, since their risk for developing the same condition is significantly greater than that of the general population [3, 4]. We have developed a unique, simple, approach for recruiting families of patients with cardiovascular disease. Our purpose is generally directed at conducting genetic studies; however, the family tree represents a valuable patient care tool in terms of prevention. Those family members found to be at risk are made aware of the necessity for routine follow-up visits, while those found to have no added risk can be reassured and spared costly periodic health evaluations.

Key words: family history, genotype, phenotypization, oligonucleotide ligation assay, hypertension.

METHODS

Patients considered to be at high risk for coronary heart disease by their referring physicians, have been asked to participate in genetic studies to determine their own risk and the risk of their family members. Our primary emphasis has been on lipid disturbances; however, we recently expanded our recruitment to include patients with essential hypertension, patients with type II diabetes mellitus, and patients with "metabolic syndrome," even if they have not yet developed overt heart disease. Index patients are identified on the wards of the hospital and also in the outpatient clinics. The physician caring for the patient informs the patient that his condition is likely to be familial and determines whether or not the patient would be willing to participate in family studies. We have observed that patients are generally grateful to be informed that their condition may be influenced by genetic factors and that they are easily motivated to recruit other family members. The family studies have been approved by the university's committee on the protection of human subjects, and written, informed consent is obtained from the index patient as well as from all participating family members.

The genetic field worker works with the patient to establish the patient's medical family tree to the best of the patient's recollection. The patient is questioned regarding all of his relatives. The number of family members and their relationship is determined, their age, gender, date of birth, or date of death if relevant are

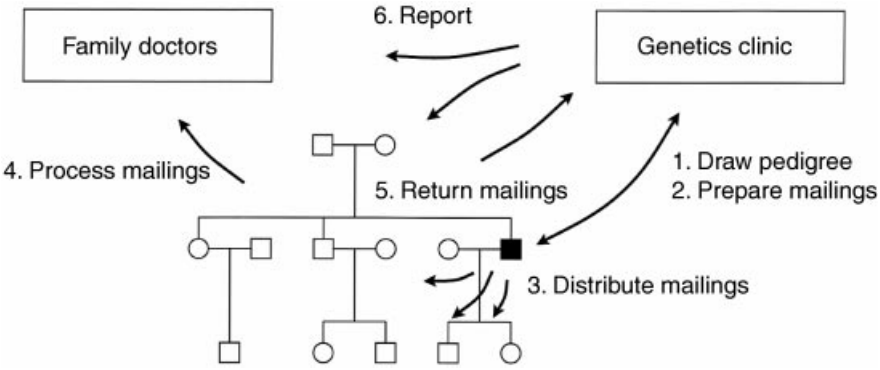

Fig. 1. Schematic illustration of the genetic field working concept. The individual steps, such as pedigree drawing, mailings, and analyses are numbered. From a practical standpoint, these steps are all computerized.

recorded. The date of any cardiovascular events (stroke, myocardial infarction, documented angina pectoris) or any known cardiovascular risk factor for each family member is determined. The primary contact with the patient's relatives is organized by the index patient himself. The index patient is given a packet for each relative containing an explanatory letter, an informed consent statement to be signed, a questionnaire, a booklet concerning risks for cardiovascular disease and the nature of family studies, and two, 10 ml blood cuvettes anticoagulated with EDTA. The index patient mails the packets or delivers them to his relatives personally. All relatives agreeing to participate sign the consent form, complete the questionnaire, and take the blood cuvettes to their family physician after having fasted overnight. The physician records the subject's blood pressure height and weight and draws the blood. We require the subjects to mail the preaddressed packets back to us, thereby documenting the voluntary nature of their participation.

In the department of clinical genetics, the blood is separated, DNA is extracted from the cells, and the serum is analyzed for total cholesterol, high density lipoprotein (HDL)-cholesterol, low density lipoprotein (LDL)-cholesterol, triglyceride concentrations and Lp(a). Data are transferred from the questionnaire and from the laboratory into a computer program. Participating family members receive their own laboratory results, as well as a brief interpretation. Family members whose values warrant attention are motivated to seek counseling with the help of their physicians. Furthermore, each individuals family physician is informed as well. To maintain confidentiality for other family members, only the family physician receives a copy of the complete family tree, albeit without the names and dates of birth of family members not under his or her care. All participants whose values are abnormal or borderline, are automatically sent a follow-up questionnaire. They are questioned with regards to the results of any follow-up tests or treatment given. In this way, we seek to motivate the subjects further by emphasizing the important nature of our activities. The entire concept is illustrated in Figure 1. The individual steps, such as pedigree drawing, mailings, and analyses are numbered. From a practical standpoint, these steps are all computerized. Our study units for genetic approaches are shown in Figure 2. Multigenerational pedigrees require in general three generations and more than 10 members. Sibling pairs are either both affected or discordant. If DNA samples are available from both parents (complete set), identity by descent can be determined. DNA from one parent is very helpful, particularly if DNA from other family members is available. If no DNA from parents is available, the identity by descent status cannot be determined unequivocally in all cases. Comparison of genotypes in this case is based on identity by state. Trios permit parental control studies. Ideally, the index patient and both parents are recruited. However, if one parent is missing, the genotype of that parent may be deduced by genotyping close relatives.

Our eventual purpose is to couple genetic field working with DNA-based diagnostic tests. For familial hypercholesterolemia, we have developed an oligonucleotide ligation assay, which effectively identifies 22 LDL receptor mutations identified in German familial hypercholesterolemia patients. The assay can be increased in capacity fivefold. The technical details of the assay are described elsewhere [8].

RESULTS

Currently, our entire database comprises 16,869 individuals from 1137 families of at least three or more members. Index patients were overwhelmingly receptive, willing, and cooperative, although some persons we contacted were unable to cooperate because they were orphaned, had no contact with family members, the family was not in Germany or for other personal reasons. The family size was highly variable. The smallest family size included was defined as three individuals consisting of a set of parents and one child. The largest family we were able to recruit consisted of 86 family members. The average family size contained 15 individuals. This value included known relatives both dead and alive. The largest family provided information on 86 persons. From the total number of 12,566 living relatives, we were able to obtain questionnaires and blood samples from 2730 persons (22%). Thus, each participating patient recruited an average of 2.4 additional relatives.

Table 1 shows the numbers of subjects we have recruited into the program. We initially concentrated solely on lipid disturbances. There are 89 families with at least 10 members with DNA samples. The total number of sibpairs comprises 1191, including 773 trios. For hypertension studies, our numbers are less. The phenotype is more difficult to procure and the parents are frequently not available. We currently have 148 sibpairs with 94 trios.

Currently, our genetic field working program is highly relevant to patients with familial hypercholesterolemia, which we can identify with the oligonucleotide ligation assay. We have used this test to screen patients with high LDL cholesterol values identified by genetic field working. We have found 32 new individuals with familial hypercholesterolemia. Each individual represents a new

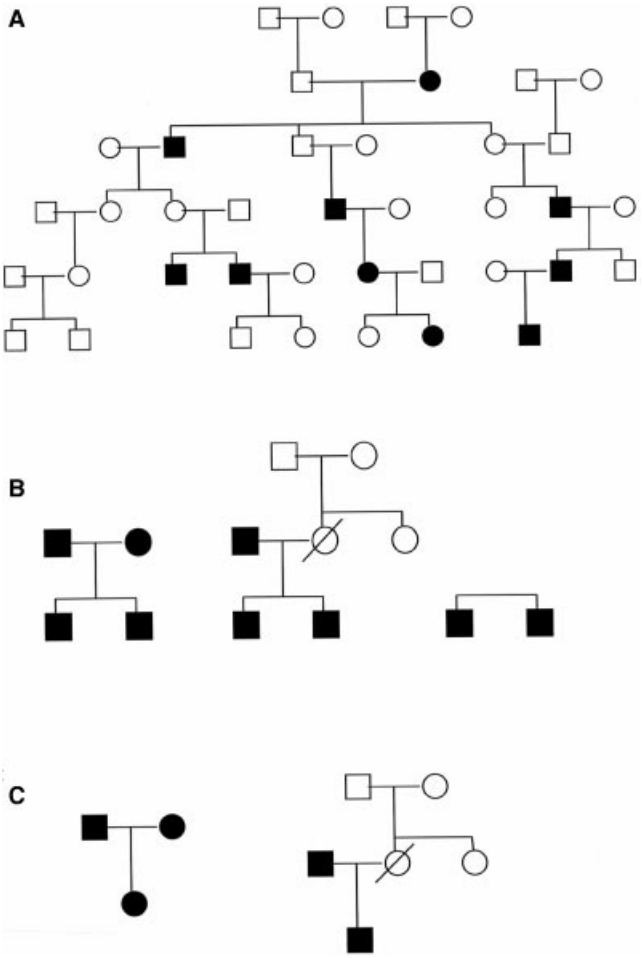

Fig. 2. Study units for genetic approaches. (*A*) Multigenerational pedigrees require in general three generations and more than 10 members. (*B*) Sibling pairs, either affected or discordant. If DNA samples are available from both parents (complete set), identity by descent can be determined. DNA from one parent is very helpful, particularly if DNA from other family members is available. If no DNA from parents is available, the identity by descent status cannot be determined. Comparison of genotypes in this case is based on identity by state. (*C*) Trios permit parental control studies. Ideally, the index patient and both parents are recruited. However, if one parent is missing, the genotype of that parent may be deduced by genotyping close relatives.

family, the affected members of which all providing an ideal opportunity for primary disease prevention. Figure 3 shows the automated genotype analysis of oligonucleotide ligation fragments from a single patient. Figure 4 shows primary representative data from one oligonucleotide ligation assay experiment with DNA from 16 patients. Table 2 outlines eight mutations found in the 32 familial hypercholesterolemia patients and in 34 family members.

DISCUSSION

We developed a program of genetic field working that is computer based. The family tree is drawn on the screen, the patient and family member data are stored in a fashion that they can be readily retrieved and analyzed, and the patient and the family members are identified so that no confusion can occur. Nevertheless, the identity of a participants is protected at every step. Furthermore, the patient's right "not to know" is also preserved.

Table 1. Recruitment of study units

Lipid project	Families (> 10 members)	Sibpairs	Twins	Trios
Compete	89	294	11	549
One parent		219	2	224
No parents		678	351	
Total		1191	364	773
Hypertension project	**Sibpairs**	**Sibpairs**	**Twins**	**Trios**
Phenotype	BP	HT	BP	HT
Complete	141	7	11	55
One parent	113	13	2	39
No parents	171	128	183	
Total	425	1248	196	94

On all subjects, both plasma lipids and DNA are available to conduct genetic studies of hyperlipidemia. Blood pressure (BP) values are available on almost all subjects. Those with the definite phenotype "hypertension" (HT) are fewer in number. Furthermore, complete sibpair sets with parents are more difficult because the parents are usually already dead.

The strategy of recruiting families from the primary care environment has the advantage of minimizing ascertainment bias, in part because of opportunistic case finding. The patients from the primary care environment are less selected than patients referred from tertiary care centers. The ascertainment bias associated with highly selected populations limits the epidemiological conclusions that can be drawn. We are aware of the fact that primary care patient recruitment also involves some ascertainment bias. However, the recruitment of patients from the primary care environment is considerably less expensive than random cohort recruitment. In addition, problems with subject confidentiality and data protection are minimized.

Since we are recruiting larger families, we are in a position of decreasing the ascertainment bias of the index subjects by including the members of the family in genetic analyses. In particular, we are able to utilize the spouses as controls, who are similar to randomly selected controls and are relatively matched for age and gender. In this fashion, one can separate families and define case and control groups for the purpose of association studies. We are also able to utilize the core families and conduct sibling-pair linkage studies on the basis of concordance or discordance (identity by descent). This approach provides the advantage of permitting both association studies and linkage analysis in the same cohort. Furthermore, we are in a position to deal with the problem of locus heterogeneity as well as the difficulty of allelic heterogeneity. These two features are particularly prominent in the genetic analysis of the complex diseases.

Studies of lipid disturbances have the advantage that the genotype and phenotype arrive in the same blood sample. Our families with familial hypercholesterolemia are an ideal example of how sensible primary prevention can result from genetic field working and DNA-based diagnostic testing. Affected family members are identified and nonaffected family members can be reassured. Familial hypercholesterolemia is an example of a genetic disease for which effective preventative treatment is available.

However, not all the families we recruited will have a familial disorder. In some families, only the index patient had elevated

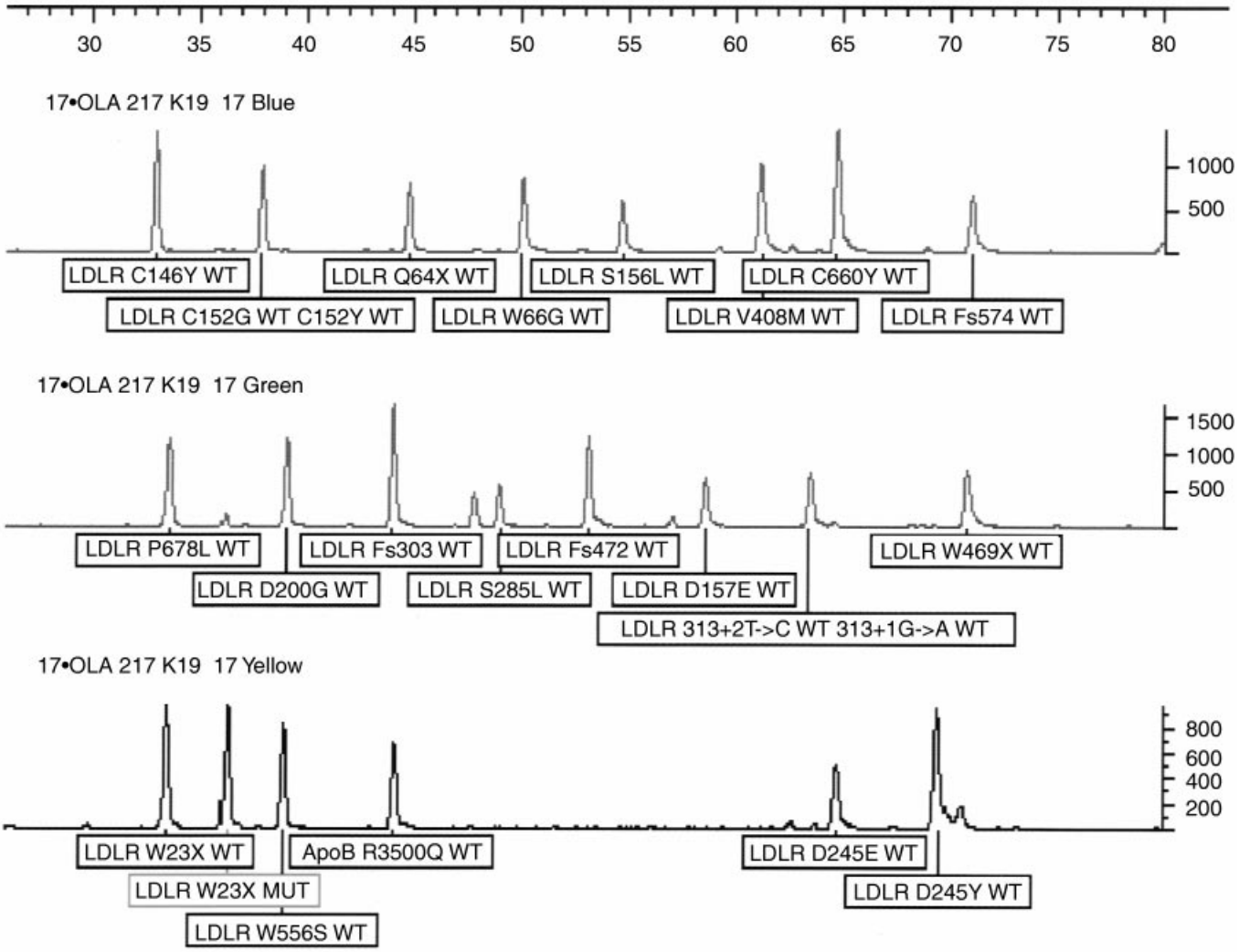

Fig. 3. Automated Genotyper analysis of OLA fragments showing representative data from a single patient.

LDL-cholesterol or triglyceride values or both. Other family members may or may not have one or the other disturbances or both. We refer to these index patients as having complex hyperlipidemia in that the mode of inheritance does not follow a simple Mendelian pattern. Some of these persons have secondary hyperlipidemia associated with type II diabetes mellitus, thyroid disorders, renal disease, or the ingestion of medications. Family members of these index patients can be reassured in terms of their cardiovascular risk. Furthermore, unnecessary subsequent laboratory testing can be avoided. We also identified index patients, who had normal or only marginally elevated lipid values. Some were found to have abnormal Lp(a) values, a genetic condition for which we have not searched routinely outside of our family studies, since it cannot be readily treated.

We have also begun to recruit hypertensive patients. This condition is more complicated, since hypertension cannot be readily diagnosed with a single measurement. Our index patients who were not receiving medications all had 24-hour blood pressure measurements obtained while in the clinic. However, the parents were frequently no longer living and accurate blood pressure measurements of other family members were not always available. We have recruited > 100 families with essential hypertension with two affected siblings within the families. From these families, we have 149 affected sibling-pairs available. Hypertension was defined as the ingestion of antihypertensive medications or ≥ 3 documented blood pressure values $> 145/95$ mm Hg before age 50 years. We have thus far relied on office blood pressure measurements obtained by the subjects' physicians. We are currently field testing the idea of sending a 24-hour ambulatory blood pressure measuring device, along with operating instructions, to selected adult participants who are not ingesting antihypertensive mediations. The device could then be returned by mail and the data retrieved in the clinic.

Although association studies are valuable for identifying disease susceptiblity loci and testing candidate genes [9], we believe that linkage analysis will prove to be more informative for finding new genes responsible for hypertension. Cases in point are the monogenic hypertensive disorders, which have been elucidated by linkage strategies [10]. Due to the multifactorial nature of hypertension, a large number of hypertensive families will be needed to detect linkage between any gene loci and variations of blood pressure. Additional phenotypic data will also need to be taken

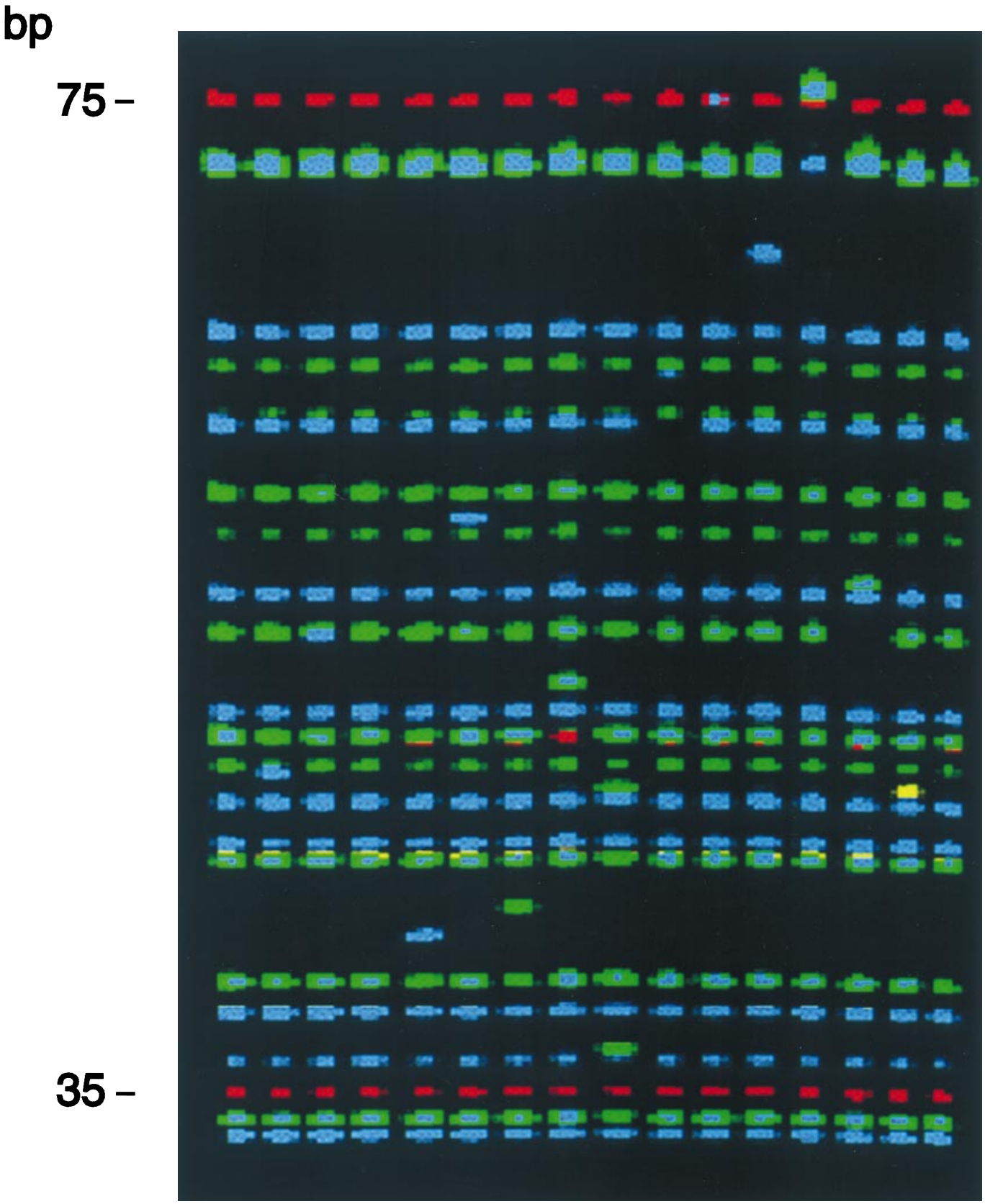

Fig. 4. Multicolor gel file showing primary data from one OLA experiment with DNA from 16 patients. Publication of this figure in color was made possible by a grant from Perkin Elmer, Applied Biosystems Division, Foster City, California, USA.

Table 2. Oligonucleotide ligation assay for familial hypercholesterolemia performed in 1076 individuals with premature coronary artery disease

Mutation	Patients	Relatives
W23X	1	11
Q64X	0	0
W66G	2	2
313+1G->A	4	1
313+2T->C	0	0
C146Y	0	0
C152G	0	0
C152Y	0	0
S156L	0	0
D157E	0	0
D200G	3	5
D245Y	0	0
D245E	6	3
S285L	0	0
Fs303	0	0
V408M	4	5
W469X	0	0
Fs472	0	0
W556S	0	0
Fs574	0	0
C660Y	0	0
P678L	1	1
ApoB R3500Q	11	6

Eight LDL-receptor mutations were identified in 32 affected persons as well as 34 asymptomatic familiy members.

into account. Particularly important is precise phenotypization of blood pressure. Our database has similarities to that described by Charru et al [11] as well as the database collected by Williams et al [4]. All databases differ in various respects, which provides an opportunity to appreciate regional, environmental, and perhaps ethnic differences between populations. Since hospital-based care and ambulatory care are held strictly separate in Germany, our approach was developed to facilitate cooperation with our unit and physicians in practice. We believe our approach is helpful in melding a cooperation between primary care physicians and researchers interested in the genetics of cardiovascular disease.

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